

What is claimed:

1. A method for multiplex primer-based amplification wherein said method comprises carrying out said amplification in a reaction mixture comprising at least a first and a second pair of target enrichment primers and at least a first pair of target amplification primers, said target  
5 amplification primers comprising a FSP and a RSP, said method comprising:
  - a. a first amplification reaction using
    - i) as a template, a nucleic acid from at least one agent, said nucleic acid containing at least one target sequence from said agent;
    - 10 ii) said first pair of target enrichment primers hybridizing to said nucleic acid and bracketing said at least one target sequence;
    - iii) said second pair of target enrichment primers hybridizing to said nucleic acid and bracketing said at least one target sequence, said second pair of target enrichment primers being located proximate to at least one target sequence and each of the second pair of target enrichment primers comprising at its 5' end a super primer binding tag  
15 corresponding to the sequence of one of the target amplification primers; and
    - iv) amplification reagents and conditions for said first amplification reaction such that the first amplification reaction generates a plurality first amplification products, wherein at least a portion of the first amplification products contain at least one complement of the super primer binding tags thereby forming at least one super primer binding site;  
20 and
  - b. a second amplification reaction using
    - i) as a template, said portion of the first amplification products containing said at least one super primer binding site;
    - 25 ii) said first pair of target amplification primers binding to said super primer binding sites on said portion of first amplification products; and
    - iii) amplification reagents and conditions for said second amplification reaction such that the second amplification reaction generates a plurality second amplification products containing the at least one target sequence.
- 30 2. The method of claim 1 where said first pair of target enrichment primers comprises a  $R_o$  and a  $F_o$  primer, said second pair of target enrichment primers comprises a  $F_i$  and a  $R_i$  primer and said first pair of target amplification primers comprises a FSP and a RSP.
3. The method of claim 2 where said super primer binding tag on  $F_i$  is identical to the sequence of the FSP such that the FSP binds the complement of the super primer binding tag on said

$F_i$  primer and the super primer binding tag on  $R_i$  is identical to the sequence of the RSP such that the RSP binds the complement of the super primer binding tag on said  $R_i$  primer.

4. The method of claim 1 where the length of each of the first pair of target enrichment primers is selected from the group consisting of: 10-40 nucleotides, 10-30 nucleotides and 10-20 nucleotides.
5. The method of claim 1 where the length of each of the second pair of target enrichment primers is selected from the group consisting of: 10-40 nucleotides, 10-30 nucleotides and 10-20 nucleotides.
6. The method of claim 1 where the length of each of the first pair of target enrichment primers is 10-20 nucleotides and the length of each of the second pair of target enrichment primers is 30 to 40 nucleotides.
7. The method of claim 1 where the length of each of the first pair of target amplification primers is 10-20 nucleotides and the length of each of the second pair of target enrichment primers is 30 to 40 nucleotides.
8. The method of claim 1 where the target enrichment primers are present at a low concentration and the target amplification primers are present at a high concentration.
9. The method of claim 8 where said low concentration is a concentration of 0.002  $\mu\text{M}$  to 0.2  $\mu\text{M}$  and said high concentration is a concentration of 0.2  $\mu\text{M}$  to 1.0  $\mu\text{M}$ .
10. The method of claim 1 where the target enrichment primers are present at a concentration that is not sufficient for exponential amplification of the target sequence and the target amplification primers are present at a concentration that is sufficient for exponential amplification of the target sequence.
11. The method of claim 1 where each of the target enrichment primers is used at the same concentration.
12. The method of claim 1 where at least one of the target enrichment primers is used at a higher concentration than the other target enrichment primers.
13. The method of claim 1 where each of the target amplification primers is used at the same concentration.
14. The method of claim 1 where at least one of the target amplification primers is used at a higher concentration than the other target amplification primer.
15. The method of claim 14 where said target amplification primer at said higher concentration comprises a means for detection.
16. The method of claim 1 where the conditions for said first amplification reaction comprise at least two complete cycles of a target enrichment process and the conditions for said second

amplification process comprise at least two complete cycles of a target amplification process.

17. The method of claim 16 where the target enrichment process comprises the following conditions for amplification: 0.5 to 1 minute at 92-94<sup>0</sup>C, 1-2.5 minutes at 50-55<sup>0</sup>C and 0.5 to 1 minute at 70-72<sup>0</sup>C and the target amplification process comprises the following conditions for amplification: 15 to 30 seconds at 94<sup>0</sup>C, 15 to 30 seconds at 50-55<sup>0</sup>C and 15 to 30 second at 72<sup>0</sup>C.
18. The method of claim 1 where the conditions for said first amplification reaction comprise at least two complete cycles of a target enrichment process and at least two complete cycles of a selective amplification process and the conditions for said second amplification process comprise at least two complete cycles of a target amplification process.
19. The method of claim 18 where the target enrichment process comprises the following conditions for amplification: 0.5 to 1 minute at 92-94<sup>0</sup>C, 1-2.5 minutes at 50-55<sup>0</sup>C and 0.5 to 1 minute at 70-72<sup>0</sup>C, the selective amplification process comprises the following conditions for amplification: 15 to 30 seconds at 92-94<sup>0</sup>C, 1 to 2 minutes at 70-72<sup>0</sup>C and the target amplification process comprises the following conditions for amplification: 15 to 30 seconds at 94<sup>0</sup>C, 15 to 30 seconds at 50-55<sup>0</sup>C and 15 to 30 second at 72<sup>0</sup>C.
20. The method of claim 19 where the selective amplification is biased toward the production of first amplification products containing the super primer binding sites.
21. The method of claim 19 where the length of each of the first pair of target enrichment primers is 10-20 nucleotides and the length of each of the second pair of target enrichment primers is 30 to 40 nucleotides.
22. The method of claim 1 further comprising three or more pairs of target enhancement primers.
23. The method of claim 1 further comprising two or more target amplification primers.
24. The method of claim 24 where said agent is selected from the group consisting of: a virus and a bacteria.
25. The method of claim 24 where said agent is a virus selected from the group consisting of: adenovirus, influenza A, influenza B, parainfluenza type 1, parainfluenza type 3, and respiratory syncytial virus, SARS, and enterovirus, including, coxsackie virus A, coxsackie virus B, rhinovirus, and echovirus.
26. The method of claim 24 where said agent is a bacteria is selected from the group consisting of: *Mycoplasma* species and *Chlamydia* species.
27. The method of claim 24 where said agent is selected by the appropriate design of first and second pair of target enrichment primers.

28. The method of claim 1 where at least one of said forward or reverse super primers further comprises a means for detection.
29. The method of claim 28 where said means for detection is selected from the group consisting of: chemical element, an enzymatic element, a fluorescent element, or a radiolabel element.
30. The method of claim 1 further comprising detecting said target sequence.
31. The method of claim 30 where the detection method is a direct detection method.
32. The method of claim 30 where said detection method comprises:
- providing at least one set of detection oligonucleotide, each set of detection oligonucleotide having a nucleotide sequence capable of binding a specific target sequence in said amplification products and comprising a first means for signal generation;
  - contacting and incubating said detection oligonucleotides with said second amplification products;
  - stimulating said first means for signal generation to produce a first signal; and
  - detecting said first signal.
33. The method of claim 32 where said first signal is unique for said agent and said first signal is used to identify said agent.
34. The method of claim 32 where said means for first signal generation is a fluorescent label, a chemical label, an enzymatic label, or a radiolabel.
35. The method of claim 32 where said means for first signal generation is a fluorescent microsphere.
36. The method of claim 30 where said method is an indirect detection method.
37. A method for multiplex primer-based amplification of at least one target sequence, wherein said method comprises carrying out said amplification in a reaction mixture comprising at least a first pair of target enrichment primers and at least a first pair of target amplification primers, said target amplification primers comprising a FSP and a RSP, said method comprising:
- a first amplification reaction using
    - as a template, a nucleic acid containing the at least one target sequence;
    - said first pair of target enrichment primers hybridizing to said nucleic acid and bracketing said target sequence, each of the first pair of target enrichment primers comprising at its 5' end a super primer binding tag corresponding to the sequence of one of the target amplification primers; and

- iii) amplification reagents and conditions for said first amplification reaction such that the first amplification reaction generates a plurality first amplification products, wherein at least a portion of the first amplification products contain at least one complement of the super primer binding tags thereby forming at least one super primer binding site; and
- 5                   and
- b. a second amplification reaction using
- i) as a template, said portion of the first amplification products containing said at least one super primer binding site;
- ii) said first pair of target amplification primers binding to said super primer binding sites on said portion of first amplification products; and
- 10                   iii) amplification reagents and conditions for said second amplification reaction such that the second amplification reaction generates a plurality second amplification products containing the target sequence.
38. The method of claim 37 where said first pair of target enrichment primers comprises a  $F_i$  and a  $R_i$  primer and said first pair of target amplification primers comprises a FSP and a RSP.
- 15                   39. The method of claim 38 where said super primer binding tag on  $F_i$  is identical to the sequence of the FSP such that the FSP binds the complement of the super primer binding tag on said  $F_i$  primer and the super primer binding tag on  $R_i$  is identical to the sequence of the RSP such that the RSP binds the complement of the super primer binding tag on said  $R_i$  primer.
- 20                   40. The method of claim 37 where the length of each of the first pair of target enrichment primers is selected from the group consisting of: 10-40 nucleotides, 10-30 nucleotides and 10-20 nucleotides.
41. The method of claim 37 where the target enrichment primers are present at a low concentration and the target amplification primers are present at a high concentration.
- 25                   42. The method of claim 41 where said low concentration is a concentration of 0.002  $\mu\text{M}$  to 0.2  $\mu\text{M}$  and said high concentration is a concentration of 0.2  $\mu\text{M}$  to 1.0  $\mu\text{M}$ .
43. The method of claim 37 where the target enrichment primers are present at a concentration that is not sufficient for exponential amplification of the target sequence and the target amplification primers are present at a concentration that is sufficient for exponential amplification of the target sequence.
- 30                   44. The method of claim 37 where each of the target enrichment primers is used at the same concentration.
45. The method of claim 37 where at least one of the target enrichment primers is used at a higher concentration than the other target enrichment primers.
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46. The method of claim 37 where each of the target amplification primers is used at the same concentration.
47. The method of claim 37 where at least one of the target amplification primers is used at a higher concentration than the other target amplification primer.
- 5 48. The method of claim 47 where said target amplification primer at said higher concentration comprises a means for detection.
49. The method of claim 37 where the conditions for said first amplification reaction comprise at least two complete cycles of a target enrichment process and the conditions for said second amplification process comprise at least two complete cycles of a target amplification process.
- 10 50. The method of claim 49 where the target enrichment process comprises the following conditions for amplification: 0.5 to 1 minute at 92-94<sup>0</sup>C, 1-2.5 minutes at 50-55<sup>0</sup>C and 0.5 to 1 minute at 70-72<sup>0</sup>C and the target amplification process comprises the following conditions for amplification: 15 to 30 seconds at 94<sup>0</sup>C, 15 to 30 seconds at 50-55<sup>0</sup>C and 15 to 30 second at 72<sup>0</sup>C.
- 15 51. The method of claim 37 where the conditions for said first amplification reaction comprise at least two complete cycles of a target enrichment process and at least two complete cycles of a selective amplification process and the conditions for said second amplification process comprise at least two complete cycles of a target amplification process.
- 20 52. The method of claim 51 where the target enrichment process comprises the following conditions for amplification: 0.5 to 1 minute at 92-94<sup>0</sup>C, 1-2.5 minutes at 50-55<sup>0</sup>C and 0.5 to 1 minute at 70-72<sup>0</sup>C, the selective amplification process comprises the following conditions for amplification: 15 to 30 seconds at 92-94<sup>0</sup>C, 1 to 2 minutes at 70-72<sup>0</sup>C and the target amplification process comprises the following conditions for amplification: 15 to 30 seconds at 94<sup>0</sup>C, 15 to 30 seconds at 50-55<sup>0</sup>C and 15 to 30 second at 72<sup>0</sup>C.
- 25 53. The method of claim 52 where the selective amplification is biased toward the production of first amplification products containing the super primer binding sites.
54. The method of claim 52 where the length of each of the first pair of target enrichment primers is 10-20 nucleotides and the length of each of the second pair of target enrichment primers is 30 to 40 nucleotides.
- 30 55. The method of claim 37 further comprising two or more target amplification primers.
56. The method of claim 37 where said agent is selected from the group consisting of: a virus and a bacteria.
57. The method of claim 37 where said agent is a virus selected from the group consisting of: adenovirus, influenza A, influenza B, parainfluenza type 1, parainfluenza type 3, and
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respiratory syncytial virus, SARS, and enterovirus, including, coxsackie virus A, coxsackie virus B, rhinovirus, and echovirus.

58. The method of claim 37 where said agent is a bacteria is selected from the group consisting of" *Mycoplasma* species and *Chlamydia* species.

5 59. The method of claim 37 where said agent is selected by the appropriate design of first and second pair of target enrichment primers.

60. The method of claim 37 where at least one of said forward or reverse super primers further comprises a means for detection.

10 61. The method of claim 60 where said means for detection is selected from the group consisting of: chemical element, an enzymatic element, a fluorescent element, or a radiolabel element.

62. The method of claim 37 further comprising detecting said target sequence.

63. The method of claim 62 where the detection method is a direct detection method.

64. The method of claim 62 where said detection method comprises:

- 15 a. providing at least one set of detection oligonucleotide, each set of detection oligonucleotide having a nucleotide sequence capable of binding a specific target sequence in said amplification products and comprising a first means for signal generation;
- 20 b. contacting and incubating said detection oligonucleotides with said second amplification products;
- c. stimulating said first means for signal generation to produce a first signal; and
- d. detecting said first signal.

65. The method of claim 64 where said first signal is unique for said agent and said first signal is used to identify said agent.

25 66. The method of claim 64 where said means for first signal generation is a fluorescent label, a chemical label, an enzymatic label, or a radiolabel.

67. The method of claim 64 where said means for first signal generation is a fluorescent microsphere.

68. The method of claim 62 where said method is an indirect detection method.

30 69. A method of diagnosing the presence of a disease agent in a subject, said method comprising:

- a. providing a sample from said subject in need of said diagnosis, said sample suspected of containing said disease agent;
- 35 b. isolating a nucleic acid from said sample, said nucleic acid containing a target sequence from said disease agent;

- c. subjecting said nucleic acid to the primer-based amplification method of any of claims 1 or 37;
- d. detecting said target sequence from said disease agent.

70. The method of claim 69 where disease agent is selected from the group consisting of: a virus  
5 and a bacteria.

71. The method of claim 69 where said disease agent is a virus selected from the group consisting of: adenovirus, influenza A, influenza B, parainfluenza type 1, parainfluenza type 3, and respiratory syncytial virus, SARS, and enterovirus, including, coxsackie virus A, coxsackie virus B, rhinovirus, and echovirus.

10 72. The method of claim 69 where said disease agent is a bacteria is selected from the group consisting of *Mycoplasma* species and *Chlamydia* species.

73. The method of claim 69 where the detection method is a direct detection method.

74. The method of claim 69 where said detection method comprises:

- 15 a. providing at least one set of detection oligonucleotide, each set of detection oligonucleotide having a nucleotide sequence capable of binding a specific target sequence in said amplification products and comprising a first means for signal generation;
- b. contacting and incubating said detection oligonucleotides with said second amplification products;
- 20 c. stimulating said first means for signal generation to produce a first signal; and
- d. detecting said first signal.

75. The method of claim 74 where said first signal is unique for said disease agent and said first signal is used to identify said disease agent.

25 76. The method of claim 74 where said means for first signal generation is a fluorescent label, a chemical label, an enzymatic label, or a radiolabel.

77. The method of claim 74 where said means for first signal generation is a fluorescent microsphere.

78. The method of claim 69 where said method is an indirect detection method.

30 79. A method for differentially diagnosing the presence of a disease agent and a secondary disease agent in a subject, said method comprising:

- a. providing a sample from said subject in need of said diagnosis, said sample suspected of containing said disease agent or said secondary disease agent;
- b. isolating a nucleic acid from said sample, said nucleic acid containing a target sequence from said disease agent or secondary disease agent or both;



- c. subjecting said nucleic acid to the primer-based amplification method of any of claims 1 or 38;
  - d. detecting said target sequence from said disease agent or secondary disease agent or both.
80. The method of claim 79 where disease agent or secondary disease agent is selected from the group consisting of: a virus and a bacteria.
81. The method of claim 79 where said disease agent or secondary disease agent is a virus selected from the group consisting of: adenovirus, influenza A, influenza B, parainfluenza type 1, parainfluenza type 3, and respiratory syncytial virus, SARS, and enterovirus, including, coxsackie virus A, coxsackie virus B, rhinovirus, and echovirus.
82. The method of claim 79 where said disease agent or secondary disease agent is a bacteria is selected from the group consisting of: *Mycoplasma* species and *Chlamydia* species.
83. The method of claim 79 where the detection method is a direct detection method.
84. The method of claim 79 where said detection method comprises:
- a. providing at least one set of detection oligonucleotide, each set of detection oligonucleotide having a nucleotide sequence capable of binding a specific target sequence in said amplification products and comprising a first means for signal generation;
  - b. contacting and incubating said detection oligonucleotides with said second amplification products;
  - c. stimulating said first means for signal generation to produce a first signal; and
  - d. detecting said first signal.
85. The method of claim 84 where said first signal is unique for said disease agent and secondary disease agent and said first signal is used to identify said agent.
86. The method of claim 84 where said means for first signal generation is a fluorescent label, a chemical label, an enzymatic label, or a radiolabel.
87. The method of claim 84 where said means for first signal generation is a fluorescent microsphere.
88. The method of claim 79 where said method is an indirect detection method.